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(54) Title: LENTIVIRAL VECTOR TRANSDUCTION OF HEMATOPOIETIC STEM CELLS

(57) Abstract: Disclosed are compositions and methods for making and using these compositions that are related to the transduction of hematopoietic stem cells. Disclosed are lentiviral vectors capable of transducing purified populations of hematopoietic stem cells. The compositions and methods can be used for treating diseases related to blood disorders such as sickle cell and thalassemia.

LENTIVIRAL VECTOR TRANSDUCTION OF HEMATOPOIETIC STEM CELLS

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority of United States Provisional Application Serial Number 60/164,625, filed November 10, 1999, now abandoned, which application is hereby incorporated by this reference in its entirety.

BACKGROUND OF THE INVENTION

Efficient transduction of hematopoietic stem cells is essential for a genetic treatment of many blood diseases. Non-cycling, quiescent stem cells (Weissman IL "Stem cells: units of development, units of regeneration, and units in evolution," *Cell*, 100:157-68 (2000)) are poorly transduced with retroviral vectors because breakdown of the nuclear membrane during mitosis is required for efficient retroviral integration into host chromatin (Varmus HE, et al., "Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA," Cell;11:307-19 (1977)).

Poor transduction of stem cells is also correlated with low level expression of receptors for amphotropic retroviral vectors (Orlic D, et al., "The level of mRNA encoding the amphotropic retrovirus receptor in mouse and human hematopoietic stem

cells is low and correlates with the efficiency of retrovirus transduction," Proc *Natl Acad Sci* U S A; 93:11097-102 (1996)).

Although adeno-associated viral (AAV) vectors have been shown to efficiently transduce postmitotic skeletal muscle or liver cells (Xiao X, Li J and Samulski RJ, "Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector," *J Virol*; 70:8098-108 (1996); Snyder RO, et al., "Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors," *Nat Genet*; 16:270-6 (1996); Fisher KJ, et al., "Recombinant adeno-associated virus for muscle directed gene therapy," *Nat Med*; 3:306-12 (1997)) transduction of hematopoietic stem cells is low (Ponnazhagan S, et al., "Adeno-associated virus type 2-mediated transduction of murine hematopoietic cells with long-term repopulating ability and sustained expression of a human globin gene in vivo," *J Virol*; 71:3098-104 (1997)). In addition, we have demonstrated that virally transduced genes are silenced after integration into host chromosomes (Chen WY, et al., "Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase," *Proc Natl Acad Sci* U S A; 94:5798-803 (1997)), and that the mechanism of silencing involves histone deacetylation and chromatin condensation (Chen WY and Townes TM, "Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation," *Proc Natl Acad Sci* U S A;97:377-82 (2000)).

In contrast to many retroviruses, the retroviral subfamily of lentiviral vectors efficiently transduce quiescent postmitotic cells (Naldini L, et al., "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector," Science; 272:263-7 (1996); Naldini L, et al., "Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector," *Proc Natl Acad Sci* U S A; 93:11382-8 (1996)). In a recent study, Miyoshi et al (Miyoshi H, et al., "Transduction of human CD34+ cells that mediate long-term

engraftment of NOD/SCID mice by HIV vectors," *Science*; 283:682-6 (1999)) demonstrated that human CD34+ cells are efficiently transduced by a lentiviral vector, and sustained expression of a GFP reporter gene is detected in NOD/SCID mice for 22 weeks. However, hematopoiesis in these animals is abnormal. The human cells in peripheral blood of these mice were predominantly B lymphocytes (Miyoshi H, et al., *Science*; 283:682-6 (1999)), and thus it is difficult to evaluate the maintenance of normal hematopoiesis after lentiviral transduction.

Thus a system for transducing hematopoietic stem cells without altering their pluripotency is needed. Disclosed are compositions and methods for transducing purified murine bone marrow stem cells.

Disclosed are compositions and methods that preserve normal hematopoiesis after transduction of hematopoietic stem cells and reconstitution of lethally irradiated recipient mice. Also disclosed are compositions and methods that promote expression of transduced genes after long term engraftment and in secondary transplants.

SUMMARY OF THE INVENTION

In accordance with the purposes of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to a purified population of hematopoietic stem cells comprising a viral vector, wherein the population of hematopoietic stem cells can differentiate into a normal distribution of blood cell types and wherein the vector is contained in similar percentages of all blood cell types.

Methods of transducing hematopoietic stem cells and treating disease are also disclosed.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

Figure 1 shows reconstitution of lethally irradiated mice with Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. Lethally irradiated recipient mice were transplanted with 1000 virally transduced Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells. Peripheral blood was collected from donor (diffuse hemoglobin haplotype), recipient (single hemoglobin haplotype), and transplants at indicated time points. Hemolysates were analyzed by HPLC using a 35% to 41% acetylnitrile gradient. Full reconstitution was achieved 8 weeks after transplantation. Tracings of the eluted globin products are shown. Both peaks due to the alpha and beta polypeptides are shown.

Figure 2 shows stable transduction of Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells by a lentiviral vector. Panel (A) shows a map of the lentiviral vector PCW-eGFP used in this study. A central polypurine tract (PPT) and a central terminal site (CTS) derived from a molecular clone of HIV-1 were inserted in the vector to increase transduction efficiency. A posttranscriptional regulatory element of Woodchuck hepatitis virus (WPRE) was placed downstream in the sense orientation with CMV/eGFP to increase

GFP expression. Panel (B) shows persistence of GFP expression in mononuclear cells from peripheral blood. The percentage of GFP^+ mononuclear cells is plotted as a function of the time in weeks post-transplantation. One thousand $Sca\text{-}1^+c\text{-}Kit^+Lin^-$ stem cells were transduced with the vector at an MOI of 50, 300, or 1000, and these cells were transplanted into a single, lethally irradiated mouse. Four mice were reconstituted with stem cells infected at an MOI of 300 and four at an MOI of 1000. Three mice were reconstituted with cells infected at an MOI of 50. At the time points indicated, fifty microliters of blood from each transplanted mouse was collected from the tail vein. Mononuclear cells were then isolated and analyzed for GFP expression by FACS. Starting at 16 weeks post-transplantation, some mice were sacrificed for secondary transplantation and bone marrow cell analysis. At least two mice from each group (MOI= 50, 300 or 1000) were analyzed after 20 weeks. GFP expression persisted for 20 weeks in all reconstituted mice. These data demonstrate that lentiviral vectors mediate stable transduction of stem cells which are capable of long-term reconstitution in vivo. The results also demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells.

Figure 3 shows a schematic of typical lentiviral vectors containing β hemoglobin as an exogenous gene element.

Figure 4 shows a typical lentiviral packaging system.

Figure 5 shows one example of a scheme for transduction of hematopoietic stem cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic methods, specific methods of recombinant biotechnology, or to particular reagents, as such may, of course, vary unless specifically limited. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

Disclosed is a purified population of hematopoietic stem cells comprising a viral vector, wherein the population of hematopoietic stem cells can differentiate into a normal distribution of blood cell types and wherein the vector is contained in similar percentages of all blood cell types.

Purified population of hematopoietic stem cells means that at least 2% of the cells in the population are hematopoietic stem cells. Hematopoietic stem cells can also comprise at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of the population.

Normal distribution of blood cell types means that the distribution of blood cell types in peripheral blood or bone marrow is about the distribution of B lymphocytes, T lymphocytes, macrophages, granulocytes, erythroid cells, etc., published in standard hematology text books.

Vector is contained in similar percentages in all blood cell types, meaning that when comparing the percentage of cells that contain the vector for a given cell type to the percentage of cells that contain the vector in a different cell type, the ratio of the highest percentage to the lowest percentage is not more than about 5-fold for any two cell types. The ratio can also be not more than about 4-fold, about 3-fold, and about 2-fold.

Also disclosed are purified populations of hematopoietic stem cells comprising a viral vector, wherein the population of hematopoietic stem cells can differentiate into

a normal distribution of blood cell types in vivo and wherein the vector is in similar percentages in all blood cell types.

Compositions

Vectors

As used herein, vectors are agents that transport a gene of interest into a cell without degradation in all cells and include a promoter yielding expression of the gene in the cells into which it is delivered. Vectors can be derived from either a virus or a retrovirus. Preferred viral vectors are lentiviral vectors, including but not limited to, SIV vectors, HIV vectors or a hybrid construct of these vectors, including viruses with the HIV backbone. These vectors also include first, second and third generation lentiviruses. Third generation lentiviruses have lentiviral packaging genes split into at least 3 independent plasmids. Also preferred are any viral families that share the properties of these viruses which make them suitable for use as vectors. Lentiviral vectors are a special type of retroviral vector which are typically characterized by having a long incubation period for infection.

Retroviral vectors can carry a larger genetic payload, i.e., an exogenous gene element, such as a transgene or marker gene, than other viral vectors, and for this reason are a commonly used vector. Adenovirus vectors are relatively stable and easy to work with, have high titers, and can be delivered in aerosol formulation, and can sometimes transfect non-dividing cells.

One embodiment of the disclosed vectors is a viral vector which has been engineered so as to suppress the immune response of the host organism, elicited by the viral antigens. Vectors of this type will typically carry coding regions for Interleukin 8 or 10.

In some embodiments, viral vectors contain, nonstructural early genes, structural late genes, an RNA polymerase III transcript, inverted terminal repeats necessary for replication and encapsidation, and promoters to control the transcription and replication of the viral genome. When engineered as vectors, viruses typically have one or more of the early genes removed and a gene or gene/promoter cassette is inserted into the viral genome in place of the removed viral nucleic acid. Constructs of this type can carry up to about 8 kb of foreign genetic material. The necessary functions of the removed early genes are typically supplied by cell lines which have been engineered to express the gene products of the early genes in trans.

Adenoviral Vectors

The construction of replication-defective adenoviruses has been described (Berkner et al., *J. Virology* 61:1213-1220 (1987); Massie et al., *Mol. Cell. Biol.* 6:2872-2883 (1986); Haj-Ahmad et al., *J. Virology* 57:267-274 (1986); Davidson et al., *J. Virology* 61:1226-1239 (1987); Zhang "Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis" *BioTechniques* 15:868-872 (1993)). The benefit of the use of these viruses as vectors is that they are limited in the extent to which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infectious viral particles. Recombinant adenoviruses have been shown to achieve high efficiency gene transfer after direct, *in vivo* delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma and a number of other tissue sites (Morsy, *J. Clin. Invest.* 92:1580-1586 (1993); Kirshenbaum, *J. Clin. Invest.* 92:381-387 (1993); Roessler, *J. Clin. Invest.* 92:1085-1092 (1993); Moullier, *Nature Genetics* 4:154-159 (1993); La Salle, *Science* 259:988-990 (1993); Gomez-Foix, *J. Biol. Chem.* 267:25129-25134 (1992); Rich, *Human Gene Therapy* 4:461-476 (1993); Zabner, *Nature Genetics* 6:75-83 (1994); Guzman, *Circulation Research* 73:1201-1207 (1993); Bout, *Human Gene Therapy* 5:3-10 (1994); Zabner, *Cell* 75:207-216 (1993); Caillaud, *Eur. J. Neuroscience* 5:1287-1291 (1993); and Ragot, *J. Gen. Virology* 74:501-507

(1993)). Recombinant adenoviruses achieve gene transduction by binding to specific cell surface receptors, after which the virus is internalized by receptor-mediated endocytosis, in the same manner as wild type or replication-defective adenovirus (Chardonnet and Dales, *Virology* 40:462-477 (1970); Brown and Burlingham, *J. Virology* 12:386-396 (1973); Svensson and Persson, *J. Virology* 55:442-449 (1985); Seth, et al., *J. Virol.* 51:650-655 (1984); Seth, et al., *Mol. Cell. Biol.* 4:1528-1533 (1984); Varga et al., *J. Virology* 65:6061-6070 (1991); Wickham et al., *Cell* 73:309-319 (1993)).

One type of viral vector is one based on an adenovirus which has had the E1 gene removed and these virions are generated in a cell line such as the human 293 cell line. In another embodiment both the E1 and E3 genes are removed from the adenovirus genome.

Another type of viral vector is based on an adeno-associated virus (AAV). This defective parvovirus can infect many cell types (including non-dividing cells) and is nonpathogenic to humans. AAV type vectors can transport about 4 to 5 kb and wild type AAV is known to stably insert into chromosome 19. Vectors can contain this site specific integration property. One embodiment of this type of vector is the P4.1 C vector produced by Avigen, San Francisco, CA, which can contain the herpes simplex virus thymidine kinase gene, HSV-tk, and/or a marker gene, such as the gene encoding the green fluorescent protein, GFP.

Retroviral Vectors

A retrovirus is an animal virus belonging to the virus family of Retroviridae, including any types, subfamilies, genus, or tropisms. Retroviral vectors, in general, are described by Verma, I.M., Retroviral vectors for gene transfer. In Microbiology-1985, American Society for Microbiology, pp. 229-232, Washington, (1985), which is incorporated by reference herein. Examples of methods for using retroviral vectors for

gene therapy are described in U.S. Patent Nos. 4,868,116 and 4,980,286; PCT applications WO 90/02806 and WO 89/07136; and Mulligan, (*Science* 260:926-932 (1993)); the teachings of which are incorporated herein by reference.

A retrovirus is essentially a package which has packed into it nucleic acid cargo. The nucleic acid cargo typically carries with it a packaging signal, which ensures that the replicated daughter molecules will be efficiently packaged within the package coat. In addition to the package signal, there are a number of molecules that are needed in cis, for the replication, and packaging of the replicated virus. Typically a retroviral genome, contains the gag, pol, and env genes which are involved in the making of the protein coat. It is the gag, pol, and env genes that are typically replaced by the foreign DNA that is to be transferred to the target cell. Retrovirus vectors typically contain a packaging signal for incorporation into the package coat (Psi), a sequence which signals the start of the gag transcription unit, elements necessary for reverse transcription, including a primer binding site to bind the tRNA primer of reverse transcription, terminal repeat sequences that guide the switch of RNA strands during DNA synthesis, a purine rich sequence 5' to the 3' LTR that serve as the priming site for the synthesis of the second strand of DNA synthesis, and specific sequences near the ends of the LTRs that enable the insertion of the DNA state of the retrovirus to insert into the host genome. The removal of the gag, pol, and env genes allows for about up to 8 kb of foreign sequence to be inserted into the viral genome (an exogenous gene element), become reverse transcribed, and upon replication be packaged into a new retroviral particle. This amount of nucleic acid is sufficient for the delivery of one to many genes depending on the size of each transcript. Positive or negative selectable markers can be included along with other genes in the exogenous gene element. The vectors can either contain one or more of the structural and replication elements. Typically the vector containing the exogenous gene will contain the gag sequence or a variant of the gag sequence.

Preferred retroviral vectors are retroviral vectors based on the lentivirus. Lentiviral vectors are based on the nucleic acid backbone of a virus from the lentiviral family of viruses. Examples are SIV and HIV. For example, a lentiviral vector preferably contains the 5' and 3' LTR regions of a lentivirus, such as SIV and HIV. Preferred LTR regions come from SIV and HIV. Lentiviral vectors also preferably contain the Rev Responsive Element (RRE) of a lentivirus, such as SIV and HIV. A preferred RRE is that of HIV.

Exogenous Gene Element

The disclosed vectors are designed to carry exogenous gene elements that will preferably be expressed in the cells transfected with the vector. The exogenous gene elements can be any gene of interest as long as it is capable of meeting the size requirements of about less than or equal to 8 kb. One type of exogenous gene element can be a marker gene.

This marker gene produces a product and is used to determine if the gene has been delivered to the cell and once delivered is being expressed. Marker genes can be the *E. Coli* lacZ gene which encodes β-galactosidase and green fluorescent protein (eGFP).

In some embodiments the marker may be a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hydromycin, and puromycin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two examples are: CHO DHFR$^-$ cells and mouse LTK$^-$ cells. These cells lack the ability to grow without the addition of such

nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., *J. Molec. Appl. Genet.* 1: 327 (1982)), mycophenolic acid, (Mulligan, R.C. and Berg, P. *Science* 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., *Mol. Cell. Biol.* 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puramycin.

The exogenous gene element can also include genes that are replacing or supplementing a native gene in the target cell. An example of this type of exogenous gene element is the β-globin gene. Figure 3 shows the lentiviral constructs that contain a human β-globin exogenous gene element. The construct, which contains the LCR HS2 element directly linked to a beta globin gene, can be produced as described by Ryan et al. (Ryan, T.M., Behringer, R.R., Martin, N.C., Townes, T.M., Palmiter, R.D. and Brinster, "A Single Erythroid-Specific DNase I Super-hypersensitive Site Activates High Levels of Human β-globin Gene Expression in Transgenic Mice," *Genes and Development* 3:314-323 (1989)). (see also Caterina, J. J., Ryan, T. M., Pawlik, K. M.,

Palmiter, R. D., Brinster, R. L., Behringer, R. R. and Townes, T. M. (1991) Human β-globin Locus Control Region (LCR): Analysis of the 5' HS 2 Site in Transgenic Mice. *Proc. Natl. Acad. Sci.*, 88:1626-1630.) Another example of this type of exogenous gene element is the α globin gene. The construct, which contains the LCR HS2 element directly linked to a beta globin gene, can be produced as described in the papers above. Another exogenous gene element is the anti-sickling gene. This construct can be produced as described in McCune, S.L., Reilly, M.P., Chomo, M.J., Asakura, T. and Townes, T.M. (1994) Recombinant Human Hemoglobins Designed For Gene Therapy Of Sickle Cell Disease, *Proc. Natl. Acad. Sci.*, 91, 9852-9856. Another type of exogenous gene element is a mutated EKLF which can be produced as described in Donze, D., Jeancake, P.H. and Townes, T.M. (1996) Activation of delta-globin Gene Expression by Erythroid Krupple Like Factor (EKLF): Novel Strategy for Gene Therapy of Sickle Cell Disease, *Blood* 88:4051-4057.

Viral Promoters and Enhancers

Promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. beta actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., *Nature*, 273: 113 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment (Greenway, P.J. et al., *Gene* 18: 355-360 (1982)). Of course, promoters from the host cell or related species also are useful herein.

Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5' (Laimins, L. et al., *Proc. Natl. Acad. Sci.* 78: 993 (1981)) or 3' (Lusky, M.L., et al., *Mol. Cell Bio.* 3:1108

(1983)) to the transcription unit. Furthermore, enhancers can be within an intron (Banerji, J.L. et al., *Cell* 33: 729 (1983)) as well as within the coding sequence itself (Osborne, T.F., et al., *Mol. Cell Bio.* 4: 1293 (1984)). They are usually between 10 and 300 bp in length, and they function in cis. Enhancers function to increase transcription from nearby promoters. Enhancers also often contain response elements that mediate the regulation of transcription. Promoters can also contain response elements that mediate the regulation of transcription. Enhancers often determine the regulation of expression of a gene. While many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein and insulin), typically one will use an enhancer from a eukaryotic cell virus. Examples are the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The promotor and/or enhancer may be specifically activated either by light or specific chemical events which trigger their function. Systems can be regulated by reagents such as tetracycline and dexamethasone. There are also ways to enhance viral vector gene expression by exposure to irradiation, such as gamma irradiation, or alkylating chemotherapy drugs.

The promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize expression of the region of the transcription unit to be transcribed. In some embodiments the promoter and/or enhancer region can be active in all eukaryotic cell types. A promoter of this type is the CMV promoter (650 bases). Other promoters are SV40 promoters, cytomegalovirus (full length promoter), and retroviral vector LTF.

It has been shown that specific regulatory elements can be cloned and used to construct expression vectors that are selectively expressed in specific cell types such as

hematopoietic cells. For example, the LCRs of the alpha- and beta-globin genes specifically upregulate the expression of the alpha and beta globin genes only in erythroid cells. Ryan, T.M., Behringer, R.R., Townes, T.M., Palmiter, R.D. and Brinster, R. L. (1989) "High Level Erythroid Expression of Human b-Globin Genes in Transgenic Mice," *Proc. Natl. Acad. Sci.* 86, 37-41. Thus, exogenous gene elements controlled by an LCR of either α or β globin will typically only be expressed in erythrocytes.

Post transcriptional regulatory elements

The disclosed vectors can also contain post-transcriptional regulatory elements. Post-transcriptional regulatory elements can enhance mRNA stability or enhance translation of the transcribed mRNA. A post-transcriptional regulatory sequence that works well with the disclosed vectors is the WPRE sequence isolated from the woodchuck hepatitis virus. (Zufferey R, et al., "Woodchuck hepatitis virus post-transcriptional regulatory element enhances expression of transgenes delivered by retroviral vectors," J Virol; 73:2886-92 (1999)). Post-transcriptional regulatory elements can be positioned both 3' and 5' to the exogenous gene, but it is preferred that they are positioned 3' to the exogenous gene.

Transduction efficiency elements

Transduction efficiency elements are sequences that enhance the packaging and transduction of the vector. These elements typically contain polypurine sequences. One transduction efficiency element that works well with the disclosed vectors is the ppt-cts sequence that contains the central polypurine tract (ppt) and central terminal site (cts) from the HIV-1 pSG3 molecular clone (SEQ ID NO:1 bp 4327 to 4483 of HIV-1 pSG3 clone).

3' untranslated regions

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells) may also contain sequences necessary for the termination of transcription which may affect mRNA expression. These 3' untranslated regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding the exogenous gene. The 3' untranslated regions also include transcription termination sites. The transcription unit also can contain a polyadenylation region. One benefit of this region is that it increases the likelihood that the transcribed unit will be processed and transported like mRNA. The identification and use of polyadenylation signals in expression constructs is well established. Homologous polyadenylation signals can be used in the transgene constructs. In an embodiment of the transcription unit, the polyadenylation region is derived from the SV40 early polyadenylation signal and consists of about 400 bases. Transcribed units can contain other standard sequences alone or in combination with the above sequences improve expression from, or stability of, the construct.

The disclosed population of hematopoietic stem cells can comprise a vector that comprises an exogenous gene element. Furthermore, the disclosed vectors can be lentiviral vectors and the lentiviral vectors can comprise an exogenous gene element. Also disclosed are lentiviral vectors that contain genes that encode anti-sickling proteins and EKLF proteins having altered binding specificity as the exogenous gene elements.

The disclosed viral vectors and lentiviral vectors can comprise a transduction efficiency element, such as the ppt-cts sequence, derived from for example, the HIV-1 pSG3 molecular clone.

The disclosed viral vectors and lentiviral vectors can comprise a post-transcriptional regulatory element, such as the WPRE region isolated from the

woodchuck hepatitis virus, and the WPRE region can be downstream from the exogenous gene element.

Packaging vectors

As discussed above, retroviral vectors are based on retroviruses which contain a number of different sequence elements that control things as diverse as integration of the virus, replication of the integrated virus, replication of un-integrated virus, cellular invasion, and packaging of the virus into infectious particles. While the vectors in theory could contain all of their necessary elements, as well as an exogenous gene element (if the exogenous gene element is small enough) typically many of the necessary elements are removed. Since all of the packaging and replication components have been removed from the typical retroviral, including lentiviral, vectors which will be used within a subject, the vectors need to be packaged into the initial infectious particle through the use of packaging vectors and packaging cell lines. Typically retroviral vectors have been engineered so that the myriad functions of the retrovirus are separated onto at least two vectors, a packaging vector and a delivery vector. This type of system then requires the presence of all of the vectors providing all of the elements in the same cell before an infectious particle can be produced. The packaging vector typically carries the structural and replication genes derived from the retrovirus, and the delivery vector is the vector that carries the exogenous gene element that is preferably expressed in the target cell (Figure 4). These types of systems can split the packaging functions of the packaging vector into multiple vectors, e.g., third-generation lentivirus systems. Dull, T. et al., "A Third-generation lentivirus vector with a conditional packaging system"J. Virol 72(11):8463-71 (1998)

Retroviruses typically contain an envelope protein (env). The Env protein is in essence the protein which surrounds the nucleic acid cargo. Furthermore cellular infection specificity is based on the particular Env protein associated with a typical retrovirus. In typical packaging vector/delivery vector systems, the Env protein is

expressed from a separate vector than for example the protease (pro) or integrase (in) proteins. (see Figure 4)

Packaging cell lines

The vectors are typically generated by placing them into a packaging cell line. A packaging cell line is a cell line which has been transfected or transformed with a retrovirus that contains the replication and packaging machinery, but lacks any packaging signal. When the vector carrying the DNA of choice is transfected into these cell lines, the vector containing the gene of interest is replicated and packaged into new retroviral particles, by the machinery provided in cis by the helper cell. The genomes for the machinery are not packaged because they lack the necessary signals. One type of packaging cell line are 293 cells.

Hematopoietic stem cells

The blood systems of mammals are very complex mixtures of many highly differentiated cells. The multiple different cell types which make up mammalian blood are all descended from a single type of cell, a hematopoietic stem cell. A hematopoietic stem cell is able to give rise to all of the cell types that make up blood. The hematopoietic stem cell differentiates into three main types of lineages including: lymphoid, myeloid and erythroid. B-cells and T-cells are descended from the lymphoid lineage while the myeloid lineage, gives rise to for example, monocytes, granulocytes, megakaryocytes, neutrophils, as well as other cells. The erythroid lineage produces red blood cells.

There are a number of organs which can be assayed from the presence of hematopoietic cells. For example, hematopoietic cells should be found in the peripheral blood (PB), bone marrow (BM), spleen, and thymus.

A common way to characterize a given blood cell is through antibody recognition. Different surface markers are present on each cell depending on its level of differentiation and type of differentiation.

In human hematopoietic cells the CD34 marker is known to be present on hematopoietic stem cells. This marker however is also present on a number of other cells which are more differentiated than the hematopoietic stem cell including, B-cells (CD19+ cells) and myeloid cells (CD33+ cells) which make up 80-90% of the CD34+ population.

Other markers which are present on hematopoietic cells are CD3, CD8, CD10, CD15, CD19, CD20, and CD33 and of all of these markers are assayed >90% of all CD34+ cells will be accounted for.

B cells contain among other markers CD10/19/20 markers while T-cells contain among other markers CD3/4/8 markers. Similarly CD14/15/33 cell markers can be found on myeloid cells. Human T-cells lack the Thy-1 marker. Therefore, according to United States Patent No 5,716,827, which is herein incorporated by reference, human stem cells are characterized by being for the most part $CD34^+$, $CD3^-$, $CD7^-$, $CD8^-$, $CD10^-$, $CD14^-$, $CD15^-$, $CD19^-$, $CD20^-$, $CD33^-$, and $Thy\text{-}1^+$. The more of these markers that are selected for, the more purified the hematopoietic stem cell population will be. A related United States Patent which is also herein incorporated by reference is United States Patent No. 5,061,620.

Mouse markers that can be used are Scal, c-Kit, B-220, CD3, CD4, CD5, CD8, Mac-1, Gr-1, and Ter-119 as described in Chen, W., Wu, X., Liu, H., Zhang, M, Lai, L, Ciavatta, D., Kappes, J. and Townes, T. (2000) Lentiviral transduction of murine hematopoietic stem cells that mediate long term reconstitution of lethally irradiated mice, *Stem Cells* 18:352-359.

Those of skill in the art understand that other markers differentiating hematopoietic stem cells from differentiated cells exist and they can be incorporated and used as described herein.

One way of characterizing a hematopoietic population of stem cells is that hematopoietic stem cells express proteins such as CD34+ (human) or Sca1 and c-Kit (mouse) and do not express progenitor or lineage specific proteins such as CD38, CD19, CD14, CD2, CD3, CD5, glycophorin (human) or B220, Ter119, CD3, CD4, CD5, CD8, Mac1 and GR1 (mouse). The cells can also be defined as cells that efflux specific dyes such as Hoechst 33342 and by the fluorescence emission pattern of cells stained with dyes such as Hoechst 33342. Hematopoietic stem cells with these characteristics can be obtained from bone marrow, peripheral blood or cord blood. The cells can also be derived from other cell types including muscle, neuronal cells, embryonic stem cells and embryonic gonadal cells.

Different mammals have different markers which differentiate their cognate hematopoietic cells. For example, the mouse hematopoietic stem cell can be purified by selecting for Sca1, c-Kit and against B-220, CD3, CD4, CD5, CD8, Mac-1, Gr-1, and Ter-119.

Sickle mice

The disclosed vectors and hematopoietic cells can be used within specific genetic backgrounds. One such back ground is the mouse model for sickle cell disease. The compositions and methods for making a preferred mouse model for sickle cell disease are described in Ryan, T.M., Ciavatta, D. and Townes, T.M. (1997) Knockout/Transgenic Mouse Model of Sickle Cell Disease, *Science*, 278: 873-876, which is herein incorporated by reference.

It is understood that organisms, particularly mammals, other than humans, that have been modified and reconstituted with the populations of hematopoietic stem cells herein disclosed are within the scope of this invention. For example a mouse carrying the transfected hematopoietic stem cells is deemed within the scope of this invention.

Methods of making the compositions

Making the vectors

The disclosed viral vectors can be made using standard recombinant molecular biology techniques. Many of these techniques are illustrated in Maniatis (Maniatis et al., "*Molecular Cloning*--A Laboratory Manual," (Cold Spring Harbor Laboratory, Latest edition) and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.

In certain cases the exogenous gene elements themselves are constructs which have been modified through recombinant biotechnology. For example an anti-sickle gene is a modified hemoglobin gene which competitively inhibits the polymerization of sickle hemoglobin. Preferred anti-sickling genes to be used as exogenous gene elements in the present compositions are disclosed and described as well as methods of making and using these preferred anti-sickling genes in McCune, S.L., Reilly, M.P., Chomo, M.J., Asakura, T. and Townes, T.M. (1994) Recombinant Human Hemoglobins Designed For Gene Therapy Of Sickle Cell Disease, Proc. Natl. Acad. Sci., 91, 9852-9856. which are specifically herein incorporated by reference.

The modified EKLF gene is a modified EKLF gene which eliminates the concatenation of sickle hemoglobin. Preferred modified EKLF genes to be used as exogenous gene elements in the present compositions are disclosed and described as well as methods of making and using these preferred EKLF genes which are specifically herein incorporated by reference.

Methods of making packaged retroviral and lentiviral packaging systems are well known.

Prior to transducing the hematopoietic stem cells it is preferred that the hematopoietic stem cells are highly purified. The hematopoietic stem cells can be purified by for example, labeling cells collected from the bone marrow of a donor with an antibody which recognizes a protein present on a hematopoietic stem cell. Then these labeled antibody-cell conjugates can be pre-purified on a column that recognizes the antibody directly or recognizes a conjugate attached to the antibody, such as biotin or streptavidin. In this type of procedure the flowthrough from the column can be discarded (or repurified to maximize recovery) and the eluate containing the antibody-hematopoietic stem cell conjugate is collected. This eluate can then be incubated with a variety of fluorescently labeled antibodies that are specific for differentiated hematopoietic cells such as antibodies to CD19 for B-cells. After incubation an incubation mix is created and this incubation mix can be sorted by FACS, wherein cells possessing the antibody which recognizes hematopoietic stem cells are collected but cells labeled with the non-hematopoietic stem cell antibodies are discarded. This type of purification protocol is described in Example 1 for murine hematopoietic stem cells, but it is readily usable for example in the purification of human hematopoietic stem cells.

Disclosed are methods of transducing a purified population of hematopoietic stem cells with a viral vector producing at least one transduced hematopoietic stem cell, comprising incubating the vector and the purified population of hematopoietic stem cells for a length of time which does not cause the hematopoietic stem cell to alter its pluripotency.

The length of time which does not cause the hematopoietic stem cells to alter their pluripotency is typically a short incubation time. For example, the length of time

is preferably less than or equal to four hours. It is understood that under the appropriate conditions, such as changing multiplicities of infection, the optimal length of time can change.

For example, in one embodiment the population of hematopoietic stem cells is incubated with a viral vector for a period of time which does not alter the stem cell pluripotency and the incubation is done with a sufficient number of vectors to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency.

Also disclosed are methods of transducing a purified population of hematopoietic stem cells with a viral vector producing at least one transduced hematopoietic stem cell, comprising incubating the vector and the purified population of hematopoietic stem cells with a sufficient number of vectors to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency.

One way to judge whether the number of vectors is sufficient to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency is to compare the number of viral vectors to the number of cells to be transduced, such as the purified population of hematopoietic stem cells. This is called a multiplicity of infection (MOI). For example, if there were three viral vectors for each cell to be transduced, this would be MOI of three. Typically the amount of vector to cell, or MOI, should be greater than or equal to about 50 times as many vectors as cells in the purified population of cells. Other examples are MOIs of 300 or 1000.

One embodiment of the disclosed methods involves incubating the vector and the purified population of hematopoietic stem cells with a sufficient number of vectors to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency, as well as incubating the vector and the purified population of hematopoietic stem cells for a length of time which does not cause the hematopoietic

stem cell to alter its pluripotency and amplifying the transduced hematopoietic cell in vivo.

Methods of using the compositions

The disclosed compositions can be used to test and validate mouse models that mimic blood disorders.

The disclosed compositions can be used to treat blood disorders.

Disclosed are methods of treating sickle cell disease in a first subject comprising incubating a population of hematopoietic stem cells purified from the first subject's bone marrow with a viral vector comprising an exogenous gene element capable of treating sickle cell disease, forming an incubation mixture, and supplying a subset of the cells from the incubation mixture for introduction to the first subject, wherein the first or second subjects have a reduced hematopoietic cell count. The first subject's incubation mix can also be introduced into a second subject having sickle cell disease assuming issues of graft versus host disease can be overcome by, for example, immune suppression.

Also disclosed are methods of treating sickle cell disease wherein the exogenous element is an anti-sickling gene and/or EKLF protein with altered binding specificity.

Also disclosed are methods of treating sickle cell disease wherein the viral vector is lentiviral vector.

Also disclosed are methods of treating a thalassemia in a first subject comprising incubating a population of hematopoietic stem cells purified from the first subject's bone marrow with a viral vector comprising an exogenous gene element

capable of treating the thalassemia, forming an incubation mixture and supplying a subset of the cells from the incubation mixture for introduction to the first subject or a second subject wherein the first subject has a reduced hematopoietic cell count.

Again, the first subject's incubation mixture could also be introduced into a second subject to treat the second subject's thalassemia.

Disclosed are methods treating a thalassemia, wherein the exogenous gene element is either the alpha globin or beta globin gene.

Also disclosed are methods for treating a thalassemia, wherein the viral vector is a lentiviral vector.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

EXAMPLES

Example 1.

Purification of murine stem cells

C57Bl/6 Hbb^d donor mice were obtained from the Jackson Lab and bred in our mouse facility. Bone marrow was flushed from femurs and tibia of 8 to 16-week old donor mice with IMDM medium containing 5 mM EDTA, 2% fetal bovine serum

(FBS) and antibiotics. After washing once with separation buffer (PBS containing 5 mM EDTA and 0.5% charcoal treated BSA), cells were labeled with biotin-conjugated Sca-1 antibody (Pharmingen) in label buffer (PBS containing 5 mM EDTA) for 15 min on ice. Cells were then washed once with separation buffer to remove free Sca-1 antibody, and labeled with magnetic bead-conjugated streptavidin (Miltenyi) for 15 min on ice. This step was immediately followed (without washing) by addition of FITC-conjugated streptavidin (Caltag) for another 15 min on ice. After labeling, cells were washed once with separation buffer, and magnetic bead-labeled cells were enriched using a MACS column (Miltenyi) as the manufacturer suggested. Cells were eluted from the column, pelleted, and resuspended in labeling buffer. Cells were then simultaneously labeled with APC-conjugated c-Kit antibody (Pharmingen) and a cocktail of PE-conjugated lineage antibodies containing B-220, CD3, CD4, CD5, CD8, Mac-1, Gr-1, and Ter-119 (Pharmingen) for 15 min on ice. Cells were washed once with separation buffer and resuspended in IMDM medium for sorting on a Becton-Dickinson FCASVantage SE. Sca-1$^+$c-Kit$^+$Lin$^-$ cells were collected into a 5-ml tube with IMDM containing 1% FBS for transduction.

Production of lentiviral GFP expressing vector

To construct the pPCW-eGFP gene transfer vector, a PCR amplified DNA fragment containing the EGFP gene (derived from pEGFP-C1, Clontech Laboratories) was ligated into the BamHI/XhoI sites of the pHR-CMV-LacZ plasmid (Naldini L, et al., Science; 272:263-7 (1996)), generating pHR-CMV-eGFP. Then, a 150 bp sequence of DNA (coordinates 4327 to 4483) containing the central polypurine tract (PPT) and central terminal site (CTS) was PCR amplified from the HIV-1 pSG3 molecular clone (Ghosh SK, et al., "A molecular clone of HIV-1 tropic and cytopathic for human and chimpanzee lymphocytes," *Virology*;194:858-64 (1993)). and ligated into the unique ClaI site of pHR-CMV-eGFP. To increase eGFP expression in the transduced cells, a post transcriptional regulatory element derived from the woodchuck hepatitis virus (WPRE) was inserted downstream of eGFP, generating the pPCW-eGFP gene transfer

vector.

Transduction of stem cells

Sorted Sca-1$^+$c-Kit$^+$Lin$^-$ stem cells were centrifuged at 300 x g for 10 min, and resuspended in IMDM medium containing 10 µg/ml dextran sulfate and 1% FBS. One thousand stem cells were infected in a total volume of 100 µl for 4 hours at 37°C and transplanted into a single, lethally-irradiated mouse as described below.

Transplantation

The congenic recipient mice (C57Bl/6 Hbb^s) were purchased from the Jackson Lab and maintained in our transgenic facility. Mice were lethally irradiated with 1250 RADS in two doses of 625 RADS each with a Picker Cyclops Cobalt-60 unit. Anesthetized mice were transplanted with 1000 stem cells per mouse in 100 µl IMDM medium by retro-orbital injection. Transplants were maintained on antibiotic water containing 1.1 g/l neomycin sulfate (Sigma) and 1x10^6 units/l polymyxin B sulfate (Sigma) for two months posttransplantation. For secondary transplantation, five million unfractionated bone marrow cells from primary transplants were retro-orbitally injected into each recipient mouse. Hematopoietic recovery of transplants was monitored by analysis of diffuse hemoglobin using high performance liquid chromatography as described previously (Ryan TM, et al., "Knockout-transgenic mouse model of sickle cell disease," *Science*; 278:873-6 (1997)).

Mononuclear cell GFP analysis

Fifty microliters of peripheral blood from each mouse was collected from the tail vein and mixed with 1 ml PBS containing 2.5 mM EDTA. The cells were further diluted to 3 ml with PBS immediately before gradient separation. Three milliliters of Histopaque-1077 (Sigma) was loaded into a 15 ml conical tube, and 3 ml of diluted blood cells were carefully layered on the top. Cells were centrifuged at 300 x g for 10 min. The opaque layer of mononuclear cells that formed at the interface was carefully

transferred to a new tube and washed once with PBS. Cells were then aliquoted and labeled with PE-conjugated antibodies as described above, and GFP expression was analyzed using FACS. The same gradient procedure was also used to prepare bone marrow mononuclear cells for analysis. Single cell suspensions of spleen or thymus were used directly for labeling and analysis.

Progenitor assay

Bone marrow cells were mixed with methylcellulose medium M3434 (Stem Cell Technology) to 3 x 10^4/ml, plated onto 35 mm plates, and cultured at 37^{0}C for 12 days as the manufacturer suggested. CFU-GEMM colonies were examined using an inverted microscope, and fluorescent images from the colonies were captured using an Olympus IX70 inverted microscope with epifluorescence optics and a Hamamatsu CCD camera.

Example 2.

Reconstitution of lethally irradiated mice with Sca-1$^+$c-Kit$^+$Lin$^-$ cells

Bone marrow was isolated from femurs and tibias of C57Bl/6 donor mice containing the diffuse hemoglobin (Hbb^d) haplotype. No 5-fluorouracil was administered to the mice to mobilize stem cells prior to marrow isolation. Sca-1$^+$c-Kit$^+$Lin$^-$ cells were isolated as described in Example 1 and transplanted into lethally-irradiated, wild-type C57Bl/6 recipient mice containing the single hemoglobin (Hbb^s) haplotype. As few as 50 of these highly purified cells were capable of fully reconstituting hematopoiesis (data not shown). Reconstitution with donor stem cells was followed by high performance liquid chromatography (HPLC) of hemolysates. Typically 1000 Sca-1$^+$c-Kit$^+$Lin$^-$ cells per recipient mouse were routinely infected with a lentiviral vector prior to transplantation. Figure 1 illustrates reconstitution in a representative animal transplanted with 1000 transduced stem cells. Within 8 weeks post-transplantation, all erythroid cells were derived from the donor as indicated by the replacement of HbbS (β^s, β^s) with HbbD (βmaj, βmin).

Design of the lentiviral vector

The lentiviral vector used in this study was a human immunodeficiency virus-1 vector pseudotyped with vesicular stomatitis virus G (VSV-G) glycoprotein. The vector contained a cytomegalovirus (CMV) promoter driving a GFP reporter gene, a central polypurine tract (PPT) and central terminal site (CTS) derived from a molecular clone of HIV-1 to increase packaging and transduction efficiency (Ghosh SK, et al., *Virology*;194:858-64 (1993)), and a posttranscriptional regulatory element derived from Woodchuck hepatitis virus (WPRE) (Figure 2A). Zufferey et al. (Zufferey R, et al., *J Virol*; 73:2886-92 (1999)) recently demonstrated that the WPRE enhances retroviral and lentiviral transduction efficiency in cultured cells by increasing the efficiency of RNA processing. Lentiviral vectors with or without WPRE were able to transduce cultured murine erythroleukemia (MEL) cells efficiently (data not shown). WPRE efficiently transduced bone marrow stem cells.

Transduction of Sca-1$^+$c-Kit$^+$Lin$^-$ cells and persistent expression of vector-derived GFP in hematopoietic lineages in vivo

Although lentiviral vectors are able to transduce quiescent cells (Naldini L, et al., *Science*; 272:263-7 (1996); Naldini L, et al., *Proc Natl Acad Sci* U S A; 93:11382-8 (1996)), transduction efficiency is enhanced when cells are induced to enter the cell cycle (Sutton RE, et al., "Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent," *J Virol*; 73:3649-60 (1999); Park F, et al., "Efficient lentiviral transduction of liver requires cell cycling in vivo," *Nat Genet*; 24:49-52 (2000)). However, induction of hematopoietic stem cell replication in vitro may result in loss of pluripotency. Therefore, purified stem cells were incubated for only 4 hours in the absence of cytokines. Cells were then transplanted into lethally-irradiated recipients in which stem cells could home to the marrow and replicate *in vivo* under conditions that favor maintenance of pluripotency. Transduction efficiency was measured by FACS analysis for GFP expression.

Figure 2B illustrates the results of GFP expression in peripheral blood mononuclear cells of mice at 5 to 20 weeks posttransplantation. These animals received stem cells transduced at MOIs of 50, 300 or 1000. Full reconstitution with donor stem cells was achieved at 8 weeks (data not shown). At an MOI of 50, an average of 4% of peripheral blood mononuclear cells were GFP positive, and the percentage was increased to 6% and 8% with MOIs of 300 and 1000, respectively. Few GFP positive mononuclear cells were detected when stem cells were transduced with an MOI of 5 (data not shown). These results demonstrate that high MOIs are required for efficient lentiviral transduction of hematopoietic stem cells. The data also demonstrate that this lentiviral vector mediates stable transduction of stem cells that are capable of long-term reconstitution in vivo.

To show that transduced hematopoietic stem cells maintained pluripotency after long term reconstitution, mice were sacrificed at 16 and 20 weeks posttransplantation and mononuclear cells were labeled with PE-conjugated, lineage-specific antibodies for B cells (α-B220), T cells (α-CD3, CD4 and CD8), neutrophils, monocytes and granulocytes (α-Mac-1 and GR-1), and erythroid cells (*a*-Ter-119). In a typical experiment at 16 weeks post-transplantation, mononuclear cells were collected from peripheral blood (A), bone marrow (B), spleen and thymus (C). This primary recipient initially received 1000 stem cells transduced at an MOI of 300. Mononuclear cells were analyzed by FACS without staining or after staining with PE-conjugated lineage antibodies (B220 for B cells, a mixture of CD 3, 4 and 8 for T cells, a mixture of Mac-1 and Gr-1 for neutrophils, monocytes and granulocytes, and Ter-119 for erythroid cells). The percentage of GFP positive mononuclear cells was determined by FACS In a typical experiment in peripheral blood, 7.9% of B cells, 9.6% of T cells, and 12.6% of neutrophils, granulocytes and monocytes were positive for GFP expression. Similar values were observed at all time points for peripheral blood mononuclear cells (data not shown). These type of results demonstrate that transduced hematopoietic stem cells maintain the capacity for normal lineage specification in fully reconstituted mice.

Less than 1% of erythroid cells in peripheral blood were positive for GFP (data not shown) due to the fact that erythroid progenitors were stably transduced but that little GFP persists in enucleated red blood cells since 9.7 % of Ter-119$^+$ bone marrow mononuclear cells (erythroid precursors) were positive for GFP expression. This demonstrated that transduced stem cells are also capable of normal erythroid lineage differentiation. Bone marrow B cells, neutrophils, granulocytes and monocytes, as well as splenic B cells and thymic T cells were all GFP positive at similar percentages. Again, these results demonstrate that lentiviral vectors efficiently transduce hematopoietic stem cells and do not alter normal properties of self-renewal and lineage specification in fully reconstituted mice.

Persistent GFP expression in secondary transplants

To determine whether GFP expression persists in secondary transplants, five million bone marrow cells from primary transplants were injected into lethally irradiated C57BL/6Hbb^d recipient mice. Table 1 illustrates one set of secondary transplants derived from a 16-week primary transplant. GFP expression was detected in all reconstituted secondary recipient mice at 12 weeks posttransplantation, and the average percentages of GFP positive mononuclear cells, B cells, T cells, and neutrophils and monocytes were consistent with those from the primary transplant. These results further support the conclusion that long term, self-renewing stem cells were transduced by the lentiviral vector and that the pluripotency of these cells was preserved in the fully reconstituted recipients.

Silencing of lentivirally transduced genes

GFP expression in CFU-GEMM (colony forming units-granulocyte, erythrocyte, macrophage, and megakaryocyte) derived from long term reconstituted mice was examined. In one typical experiment, bone marrow cells (3 x 10^5 per 35 mm plate) from fully reconstituted recipient mice at 16 weeks (mouse # TP 54) or 20 weeks (mouse # TP 62) post-transplantation were plated in methylcellulose and cultured for

12 days at 37°C. After 12 days the CFU-GEMM colonies were scored and examined by fluorescence microscopy. TP 54 and TP 62 each received 1000 stem cells that were transduced at an MOI of 1000 and 300, respectively. The percentages of GFP-positive CFU-GEMM colonies from the two recipients were analyzed and scored. GFP-positive mononuclear cells from peripheral blood (PBL) and bone marrow (BM) of the same mice were also analyzed as controls. Colonies were either fully positive or negative; no sectoring into expressing and non-expressing cells was observed. This result suggests that silencing of the transduced gene does not occur during lineage specification. However, some silencing may occur in early progenitors. The percentage of GFP positive CFU-GEMM was approximately 2 times higher than the percentage of GFP positive mononuclear cells in peripheral blood and bone marrow. Nevertheless, a high percentage of GFP positive cells persist for 16 and 20 weeks in fully reconstituted mice. Bone marrow cells were obtained from animals at 16 weeks posttransplantation and plated (3×10^4/plate in methylcellulose. As expected, the number of CFU-GEMM derived from reconstituted and wild-type mice was similar (data not shown).

In transgenic mice that express the lacZ gene specifically in erythroid cells, early erythroid colonies are sectored into lacZ expressing and non-expressing cells (Graubert TA, et al., "Stochastic, stage-specific mechanisms account for the variegation of a human globin transgene," *Nucleic Acids Res*, 26:2849-58 (1998)). This result in transgenic mice suggested that silencing of the transgene occurred during erythroid differentiation. The colonies derived from mice that were transduced with the lentiviral vector were not sectored; therefore, silencing of the transduced gene apparently does not occur during lineage specification. However, silencing may occur in stem cells or early progenitors. PCR analysis demonstrated that 40% of CFU-GEMM contained GFP DNA (data not shown); however, only 20% of these colonies and 10% of peripheral blood cells expressed GFP. These data suggest that silencing occurs at two separate stages after transduction. One half of all vector integration sites are silenced in stem cells soon after transduction. Subsequently, one half of the remaining sites are

silenced in early progenitors. Nevertheless, a high percentage (10%) of GFP positive cells persist in bone marrow and peripheral blood for 20 weeks post-transplantation in fully reconstituted mice and for at least 12 weeks in secondary recipients.

Example 3.

Highly purified murine bone marrow stem cells are efficiently transduced with a lentiviral vector and the normal pluripotency of these cells is preserved in fully reconstituted, lethally-irradiated mice. The disclosed compositions and methods maintain normal hematopoiesis after transplantation; therefore, stable lentiviral transduction does not alter normal cell lineage specification.

High multiplicities of infection (MOI) were enhanced for efficient transduction. At an MOI of 5, few cells were transduced. An MOI of 50 was required for significant transduction and an MOI of 1000 increased the efficiency of transduction two fold. The high MOIs required in the present studies may result from the use of highly purified stem cells that were transduced without cytokine stimulation. We typically used unstimulated stem cells in an attempt to preserve pluripotentiality and these undifferentiated cells may express fewer receptors for the VSV-G glycoprotein.

Efficient gene delivery into murine hematopoietic stem cells will provide a powerful tool for genetic correction of thalassemia and sickle cell disease in mouse models (Ciavatta DJ, et al., "Mouse model of human beta zero thalassemia: targeted deletion of the mouse beta maj- and beta min-globin genes in embryonic stem cells," *Proc Natl Acad Sci* U S A, ;92:9259-63 (1995); Yang B, et al., "A mouse model for beta 0-thalassemia," *Proc Natl Acad Sci* U S A; 92:11608-12 (1995); Ryan TM, et al., "Knockout-transgenic mouse model of sickle cell disease," *Science*; 278:873-6 (1997); Paszty C, et al., "Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease," *Science*; 278:876-8 (1997)) and will provide a foundation for similar protocols in humans. The life span of sickle and thalassemic red blood cells is

significantly shorter than normal; therefore, correction of approximately 10% of erythroid precursors in the marrow can translate into a major fraction in the peripheral blood. Lentivirally transduced genes can be reactivated by deacetylase inhibitors. A combination of lentiviral transduction and drug treatment can result in high level, therapeutic gene expression and thus provide a powerful treatment for hereditary blood diseases.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

Table 1 GFP expression in peripheral blood mononuclear cells of secondary transplants

	Secondary transplant							Primary transplant
	TP85	TP86	TP87	TP88	TP89	TP90	Average	TP64
No staining	10.8	12.4	14.3	8.4	4.1	9.8	10.0	11.2
B220	7.2	5.8	9.7	3.9	3.0	7.8	6.2	7.9
CD3,4&8	16.3	19.3	20.8	12.4	7.1	15.4	15.2	9.6
Mac-1/Gr-1	6.1	12.6	7.6	8.0	3.5	5.9	7.3	12.6

Five million bone marrow cells from a 16-week primary transplant were used to reconstitute lethally irradiated secondary recipients. The percentages of GFP positive cells in peripheral blood mononuclear cells, unstained or stained with lineage-specific antibodies, are shown for six secondary transplants at 12 weeks posttransplantation. The percentage of GFP positive, peripheral blood mononuclear cells in the primary recipient at 16 weeks posttransplantation is also shown for comparison.

What is claimed is:

1. A purified population of hematopoietic stem cells comprising a viral vector, wherein the population of hematopoietic stem cells can differentiate into a normal distribution of blood cell types and wherein the vector is contained in similar percentages of all blood cell types.

2. The population of hematopoietic stem cells of claim 1, wherein the population of hematopoietic stem cells differentiates into a normal distribution of blood cell types in vivo.

3. The population of hematopoietic stem cells of claim 1, wherein the vector comprises an exogenous gene element.

4. The population of hematopoietic stem cells of claim 1, wherein the vector is a lentiviral vector.

5. The vector of claim 4, wherein the vector comprises an exogenous gene element.

6. The vector of claim 5, wherein the exogenous gene element encodes an anti-sickling protein.

7. The vector of claim 5, wherein the exogenous gene element encodes an EKLF protein having altered binding specificity.

8. The vector of claim 4, further comprising a transduction efficiency element.

9. The vector of claim 8, wherein the transduction efficiency element comprises

the ppt-cts sequence.

10. The vector of claim 9, wherein the ppt-cts sequence is derived from the HIV-1 pSG3 molecular clone.

11. The vector of claim 4, further comprising a post-transcriptional regulatory element.

12. The vector of claim 11, wherein the post-transcriptional regulatory element is the WPRE region corresponding to the woodchuck hepatitis virus.

13. The vector of claim 12, wherein the WPRE region is downstream from the exogenous gene element.

14. A method of transducing a purified population of hematopoietic stem cells with a viral vector producing at least one transduced hematopoietic stem cell, comprising incubating the vector and the purified population of hematopoietic stem cells for a length of time which does not cause the hematopoietic stem cell to alter its pluripotency.

15. The method of claim 14, wherein the length of time is less than or equal to four hours.

16. The method of claim 14, further comprising incubating the vector and the purified population of hematopoietic stem cells with a sufficient number of vectors to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency.

17. A method of transducing a purified population of hematopoietic stem cells with

a viral vector producing at least one transduced hematopoietic stem cell, comprising incubating the vector and the purified population of hematopoietic stem cells with a sufficient number of vectors to achieve transduction without causing the hematopoietic stem cell to alter its pluripotency.

18. The method of claim 17, wherein the incubation includes using at least 50 times as many vectors as cells in the purified population of hematopoietic stem cells.

19. The method of claim 17, wherein the incubation includes using at least 300 times as many vectors as cells in the purified population of hematopoietic stem cells.

20. The method of claim 17, wherein the incubation includes using at least 1000 times as many vectors as cells in the purified population of hematopoietic stem cells.

21. The method of claim 20, further comprising incubating the vector and the purified population of hematopoietic stem cells for a length of time which does not cause the hematopoietic stem cell to alter its pluripotency and amplifying the transduced hematopoietic cell in vivo.

22. A method of treating sickle cell disease in a subject, comprising incubating a population of hematopoietic stem cells purified from the a subject's bone marrow with a viral vector comprising an exogenous gene element capable of treating sickle cell disease forming an incubation mixture, and supplying a subset of the cells from the incubation mixture for introduction to the subject, wherein the subject has a reduced hematopoietic cell count.

23. The method of claim 22, wherein the exogenous element is an anti-sickling gene.

24. The method of claim 22, wherein the exogenous gene element is an EKLF protein with altered binding specificity.

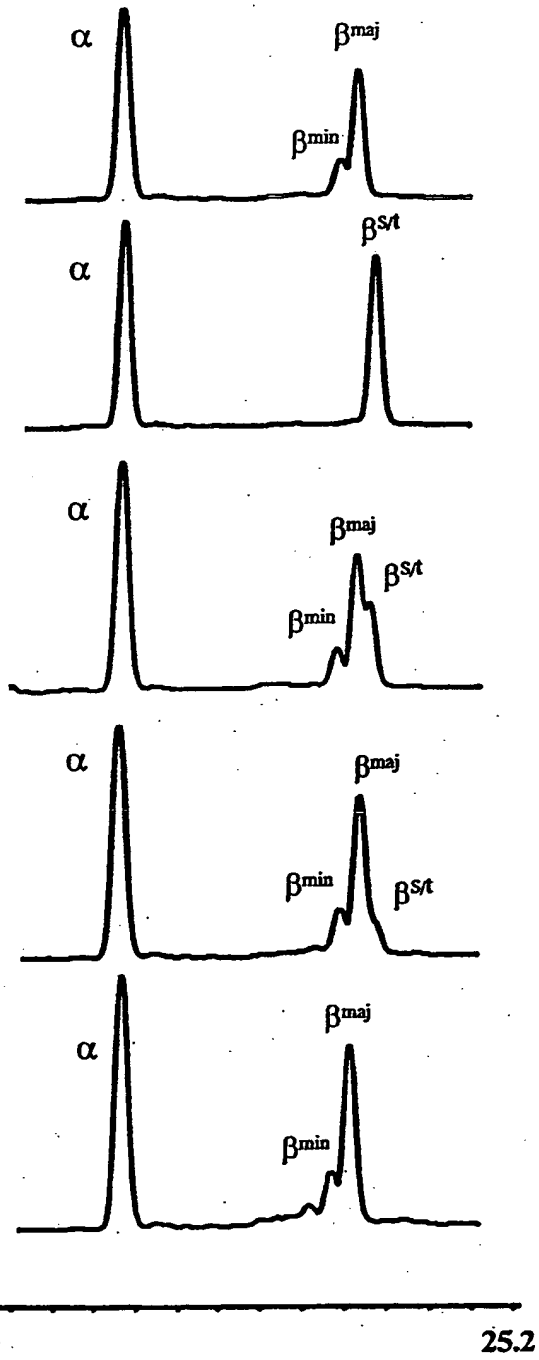
25. The method of claim 22, wherein the viral vector is lentiviral vector.

26. A method of treating a thalassemia in a subject, comprising incubating a population of hematopoietic stem cells purified from the subject's bone marrow with a viral vector comprising an exogenous gene element capable of treating the thalassemia, forming an incubation mixture and supplying a subset of the cells from the incubation mixture for introduction to the subject, wherein the subject has a reduced hematopoietic cell count.

27. The method of claim 26, wherein the exogenous gene element is either the alpha globin or beta globin gene.

28. The method of claim 26, wherein the viral vector is a lentiviral vector.

Fig. 1



Donor:
C57BL/6Hbb^d

↓ **1000 Sca$^+$c-Kit$^+$ Lin$^-$ cells transplanted**

Recipient:
C57BL/6Hbb^s

↓

3-week transplant:
60% donor-derived hemoglobin

↓

5-week transplant:
85% donor-derived hemoglobin

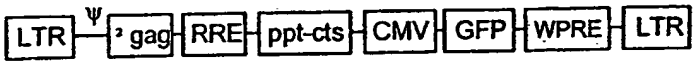
↓

8-week transplant:
100% donor-derived hemoglobin

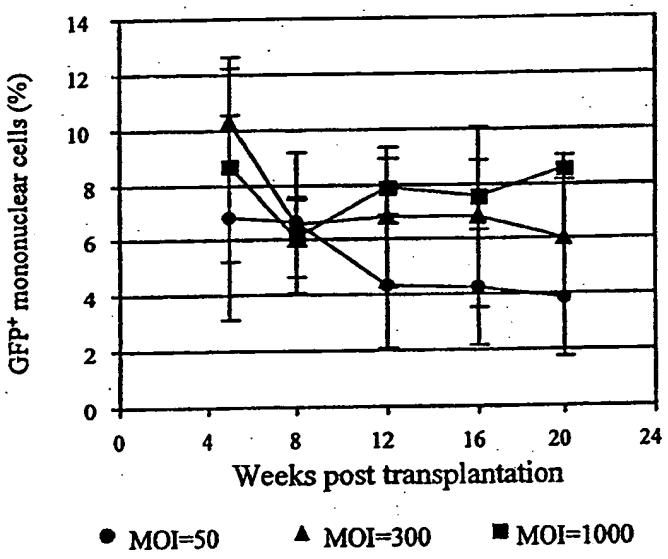
Fig. 2

A

pPCW-eGFP



B



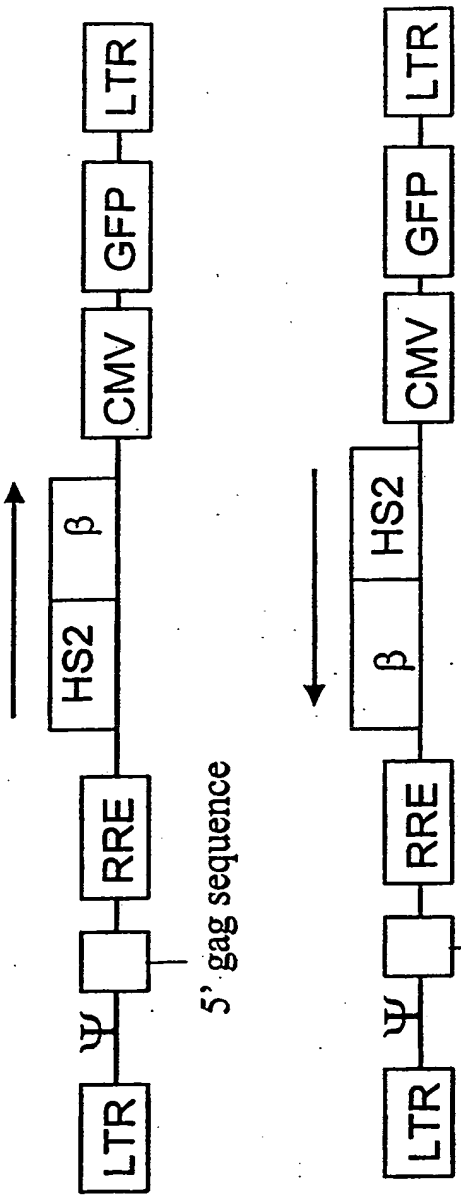


Fig. 3 HS2 β-globin gene constructs packaged into lentiviral vector

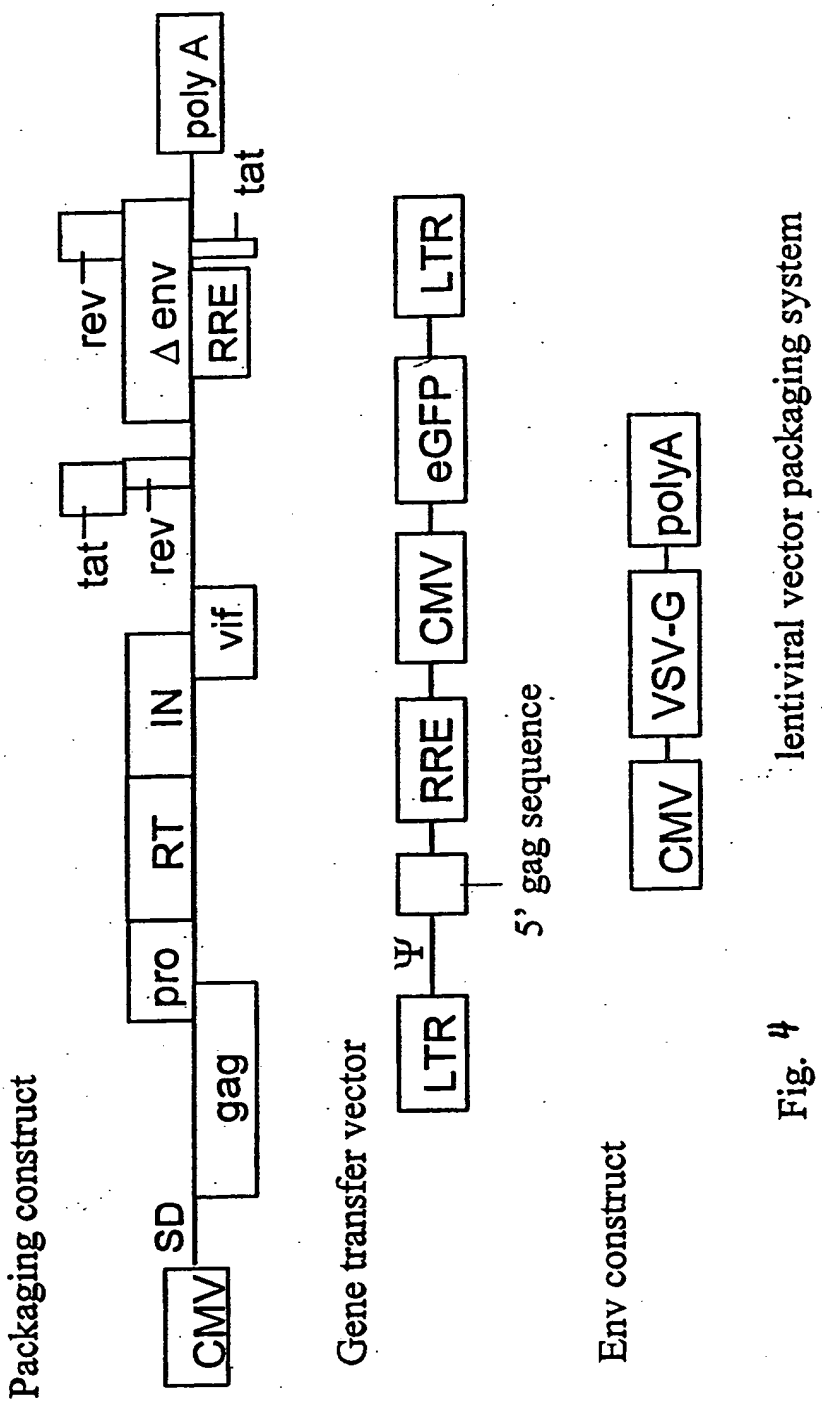


Fig. 4 lentiviral vector packaging system

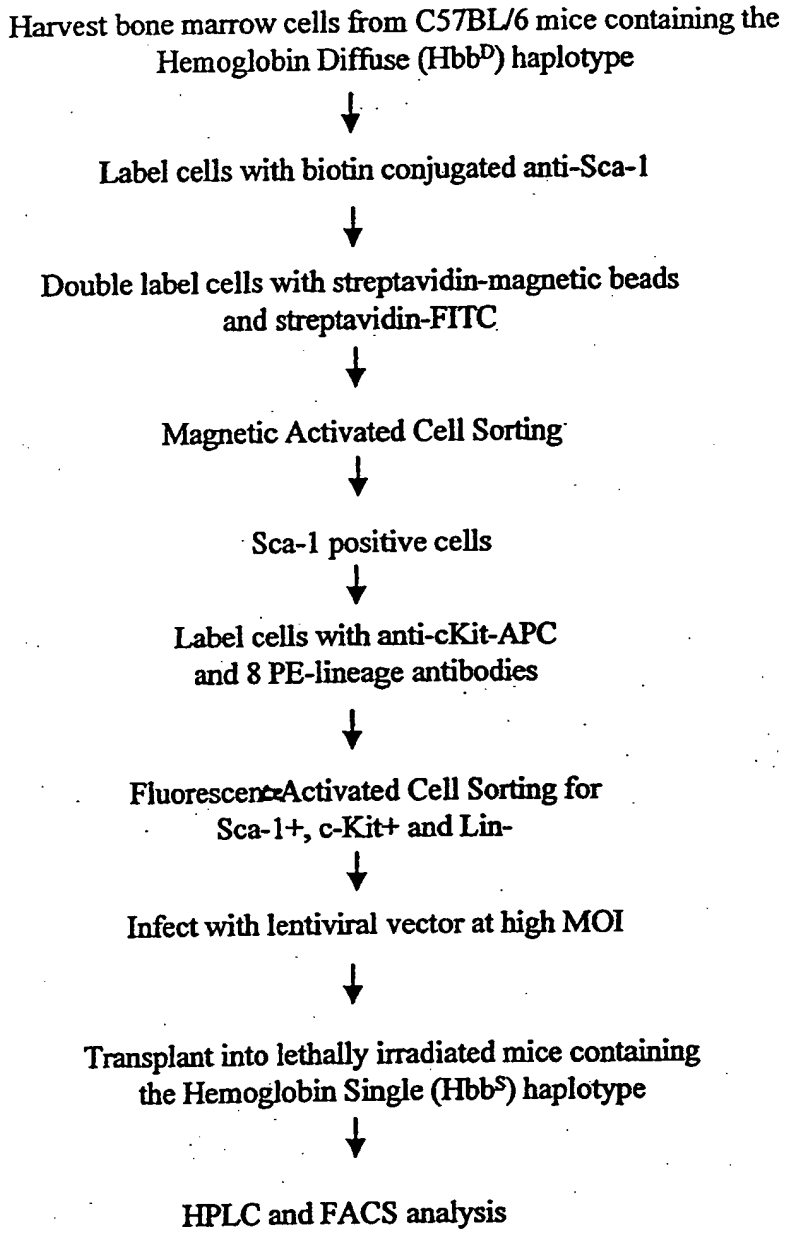


Fig. 5 Experimental scheme for hematopoietic stem cell transduction and transplantation

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/30882

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :435/6, 91.1, 320.1, 325, 375, 455; 536/23.1; 514/44; 424/93.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 320.1, 325, 375, 455; 536/23.1; 514/44; 424/93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, LIFESCI, EMBASE, USPATFULL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHEN et al. Lentiviral Vector Transduction of Hematopoietic Stem Cells that Mediate Long-Term Reconstitution of Lethally Irradiated Mice. Stem Cells. 2000, Vol. 18, pages 352-359, see entire document.	1-28
Y	MILLER et al. A Novel, Erythroid Cell-Specific Murine Transcription Factor That Binds to the CACCC Element and Is Related to the Kruppel Family of Nuclear Proteins. Molecular and Cellular Biology. May 1993, Vol. 13, No. 5, pages 2776-2786, see entire document.	5-7
Y	MCCUNE et al. Recombinant human hemoglobins designed for gene therapy of sickle cell disease. Proc. Natl. Acad. Sci., USA. October 1994, Vol. 91, pages 9852-9856, see entire document.	5, 6, 22-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 18 DECEMBER 2000	Date of mailing of the international search report 01 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN A. LACOURCIERE Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/30882

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GHOSH et al. A Molecular Clone of HIV-1 Tropic and Cytopathic for Human and Chimpanzee Lymphocytes. Virology. 1993, Vol. 194, pages 858-864, see entire document.	4, 8-10
X	DONZE et al. Activation of γ-Globin Gene Expression by Erythroid Kruppel-Like Factor: a Potential Approach for Gene Therapy of Sickle Cell Disease. Blood. 15 November 1996, Vol. 88, No. 10, pages 4051-4057, see entire document.	1-28
X ---- Y	ZIEGLER et al. Unicellular-Unilineage Erythropoietic Cultures: Molecular Analysis of Regulatory Gene Expression at Sibling Cell Level. Blood. 15 May 1999, Vol. 93, No. 10, pages 3355-3386, see entire document.	1-3 ----- 4-28
X --- Y	SUTTON et al. Transduction of Human Progenitor Hematopoietic Stem Cells by Human Immunodeficiency Virus Type 1-Based Vectors Is Cell Cycle Dependent. Journal Virology. May 1999, Vol. 73, No. 5, pages 3649-3660, see entire document.	1-5, 11, 14-21 ------- 6-10, 12, 13, 22-28
Y	ZUFFEREY et al. Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. Journal of Virology. April 1999, Vol. 73, No. 4, pages 2886-2892, see entire document.	11-13
Y	TURNER et al. Basic Kruppel-like factor functions within a network of interacting haematopoietic transcription factors. The International Journal of Biochemistry & Cell Biology. 1999, Vol. 31, pages 1169-1174, see entire document.	7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/30882

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 22-28 (in-part)
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 22-28 are directed to in vivo methods of treatment, which are considered non-statutory. Claims 22-28 have been searched based on the alleged activity of the claimed composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/30882

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12Q 1/68; C07H 21/04; C12N 15/11, 15/63, 15/85, 15/86; A61K 48/00, 35/00